*FLUOROMETRIC QUANTITATION OF FLUORESCEIN-COUPLED ANTIBODIES ATTACHED TO THE CELL MEMBRANE**

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Abstract.—A method was devised which allows the measurement, by direct and indirect fluorescence assay, of the binding of fluorescein-coupled antibodies directed against cell-membrane antigens. The systems used were: (1) species-specific antigens, (2) genetically determined isoantigens, (3) virus-determined, tumor-specific antigens, and (4) membrane-bound immunoglobulin in a Burkitt cell line. Titration of antibodies gave straight lines in the log versus log scale, the slope of the titration curve probably being dependent on the density of antigen receptors on the cell surface.

The presence on the surface of cells of either genetically determined isoantigens or tumor-specific antigens can be successfully demonstrated under the fluorescence microscope by the direct or indirect fluorescence method.[1-3] The results thus obtained have a somewhat wider applicability than those given by cytotoxicity tests, since not all cells possessing surface antigens are sensitive to the lytic effect of antibodies and complement, possibly because of a threshold effect requiring, for lysis, a minimal density of antigen receptors.[4] Fluorescence microscopy is, however, only roughly semiquantitative, the strength of the reaction being merely judged by the number of cells showing fluorescence as related to the serum dilution used.

It seemed desirable to attempt a quantitative method allowing the measure of bound fluorescent antibody. The procedure delineated in the present work involves the exposure of living cells to fluorescent antibodies—either directed against cell-surface antigens or against the immunoglobulin containing the antibodies to which the cells had previously been exposed. After the reaction, the washed cells were digested and dissolved, and the fluorescence of the resulting clear solution was measured.

Material and Methods.—(1) For direct fluorescence assay, cells from a Burkitt cell line, "Daudi," which has previously been shown[5] to react with antiserum directed against human IgM, were exposed for 20 min at 37°C to either anti-IgM fluorescent antiserum (Hyland Laboratories, Los Angeles, 30.3 mg of protein/ml, F/P ratio = 4.3) or, as a control, to anti-IgA fluorescent antiserum (Hyland Laboratories, 62.8 mg of protein/ml; F/P ratio = 3.5). Other controls were run, using both anti-IgM and anti-IgA fluorescent antisera, with other Burkitt cell lines[6] on which no IgM antigen has been demonstrated by fluorescence microscopy.

(1*a*) For the "indirect" fluorescence assay the following cells were used: biopsy cells from a reticulum cell sarcoma patient; mouse Moloney virus-induced leukemia cells grown in the ascites form (YAC and YAC-IR[4, 5] from A/Sn mice; YDAG from [A × DBA/2]F_1 mice) or as solid lymphomas (YDYA from [A.BY × DBA/2]F_1 mice); lymph node cells from A/Sn mice; mouse Moloney sarcoma virus-transformed cells grown in tissue culture (Ha2 and Ha3).[7] The size distribution of the cells was checked in a Coulter Counter Model B (100-μ aperture). When the percentage of the dead cells

exceeded 10%, the suspension was incubated for 30 min at 37°C with 0.25% trypsin. This is essential, since the fluorescent antiserum would enter the dead cells and thus increase the background fluorescence. The cells (approximately 10 million per assay when possible) were then exposed to 0.05 ml of the following sera: human isoantiserum (from a multiply transfused patient, Moulène, provided by Dr. F. Kourilsky, Hôpital Saint-Louis, Paris); mouse isoantiserum A.CA anti-A/Sn (anti-3, 4, 5, 6?, 10?, 11, 13, 14?, 23, 25, 28, 29),[8] [C57BL × DBA/2]F_1 anti-C3H (anti-1, 11, 25, 32), [C57BL × C3H] F_1 anti-DBA/2 (anti-4, 10, 13, 31); mouse anti-Moloney specific antiserum (pool from [A.BY × DBA/2]F_1 mice injected 8 months previously with 6000 r irradiated YDYA cells); heterologous (rabbit) anti-mouse serum. After 30 min at 37° the cells were washed three times with buffered special saline containing 1% gelatin, then exposed to 0.05 ml of 1:15 diluted fluorescent antiserum:goat anti-mouse γ-globulin (Hyland Laboratories, 43.2 mg of protein/ml, F/P ratio = 4.6); goat anti-rabbit γ-globulin (Microbiological Associates, Bethesda, 17.7 mg of protein/ml, F/P = 6.5); goat anti-human IgG (obtained from Professor Astrid Fagreus, Department of Immunology, Karolinska Institute, Stockholm, 15.8 mg of protein/ml, F/P = 3.7) according to the system used.

(2) After 20-min incubation at 37° the cells were washed 4 times with buffered special saline, centrifuged, resuspended in 0.1 ml of distilled water, mixed, and frozen overnight. To the thawed suspension, 0.2 ml of a solution containing 0.05 *M* Tris-HCl buffer pH 7.2, 0.002 *M* EDTA, 0.1 *M* cysteine, and 5–6 μl/ml of a 2× crystallized papain suspension (Sigma, lot 47B-1020, 29.3 mg/ml in 0.05 *M* sodium acetate; specific activity on benzoyl-L-arginine amide at pH 6.0 at 37°C: 4 μmoles/mg/min) was added with thorough mixing. The Tris buffer can be substituted by phosphate, although phosphate may, in some cases, cause clumping of the cells, which makes the subsequent digestion more difficult. The freezing and thawing in distilled water is also valuable for obtaining a good suspension during the proteolysis.

(3) The assay was incubated at 37°C; 2 hr, 3 at the maximum, were sufficient to ensure the solubilization of all the cells. Longer incubation periods result in the formation of a white precipitate, most probably cystine.

(4) 0.3 ml of 0.2 *N* NaOH was added with thorough mixing. The treatment dissolves completely any solid particles still present. According to Klugerman,[9] it does not irreversibly affect the emission properties of fluorescein–protein conjugates.

(5) 0.3 ml of 0.2 *M* H_3BO_3 was finally added to bring the pH to a value around 10.0 where the quantum yield of fluorescein-coupled proteins is maximal.[9] The solution obtained is, as a rule, clear, with no visible precipitate present. A brief (5 min) centrifugation on a table centrifuge is, however, preferable. With tissue culture cells, fine white solid particles were sometimes found floating on the surface of the supernatant, but passing the solution through a Millipore Swinnex 13 filter unit, equipped with a 0.45-μ pore size Millipore filter, was sufficient to remove them completely.

(6) The solution was then read on a Turner model 111 fluorometer equipped with a general-purpose lamp, a 7–60 primary filter, and a Wratten no. 15 secondary filter. The 110–805 microcuvettes (approximately 0.7 ml) with appropriate adaptor were used.

The suspension from the human biopsy contained many erythrocytes. Since the presence of large amounts of heme caused an extensive quenching of the fluorescence, the following modification of step 2 was adopted: The cells, after the binding of the serum and of the fluorescent antiserum as in step 1*a*, were resuspended in 4 ml of distilled H_2O and frozen overnight. To the thawed suspension, 4 ml of 0.8 saturated (at 25°C) ammonium sulfate in H_2O, adjusted to pH 7 with concentrated ammonia, were added. After 1 hr at 0°, the assays were centrifuged in a Sorvall centrifuge, model RC2-B, at 16,000 × *g* for 30 min. The red supernatant was discarded, and the white precipitate, after being washed once with 0.4 saturated $(NH_4)_2SO_4$ pH 7, was redissolved in 0.3 ml of 0.07 *M* phosphate–0.1 *M* cysteine–0.002 *M* EDTA buffer, pH 7.2, containing 1.2 μl papain suspension. The digestion was then pursued as usual. The presence at the end of the treatment of a relatively abundant white crystalline precipitate, which was easily removed by centrifugation or by filtration, did not interfere with the fluorescence measurements

which, as shown on Figure 2, gave the same results as when processed by the normal procedure.

In order to express the amount of fluorescent antibody bound to the cells not merely as arbitrary fluorescence units but as compared to the fluorescent serum used, the reading of a "blank" was subtracted from each value, and the difference (Δ) thus obtained was compared to the increase (F) in fluorescence caused by addition to the blank of a known amount (S) of the fluorescent serum. In the "direct test" (step 1) the blank was a tube containing, in the same amount as the assay, cells which, at the fluorescence microscope, had been shown to be unable to react with fluorescent serum. In the "indirect" test (step 1*a*) the blank was instead a tube from which the nonfluorescent serum, but not the subsequent fluorescent antiserum, had been omitted. The ratio $(\Delta \times S)/F$ thus indicates the amount of fluorescent serum bound by the cells present in the assay considered.

Results and Discussion.—Table 1 shows the result of two assays in the "direct" fluorescence test. About 5×10^6 cells were used for each assay. It can be seen that the binding occurs only between the anti-IgM serum and the Daudi cells, i.e., that the results obtained here confirm the findings at the fluorescence microscope.[5] It may be recalled that Neurath[10] has worked out a method whereby antigen–fluorescent antibody insoluble complexes are collected on a Millipore filter, the fluorescence of which is directly estimated. The sensitivity is, however, rather low, since relatively small particulate antigens, such as rabies virus, which have an extremely large surface/volume ratio if compared to mammalian cells, already give very low readings by that method.

The direct test has the disadvantage that different control cells could give nonidentical "blank" values. Although in our case this was not so, the possibility still exists and is a serious theoretical limitation. The use, for the blanks, of the same cell strain but with either nonbinding or nonfluorescein-coupled sera encounters practically the same difficulty, since various sera may behave in different ways, i.e., give different background values.

TABLE 1. *Specific binding of anti-IgM fluorescent γ-globulin to "Daudi" cells.*

Expt. no.	Antiserum used	$(\Delta \times S)/F$ (μl of antiserum specifically bound)
1	anti-IgA	0
	anti-IgM	+0.055
2	anti-IgA	−0.01
	anti-IgM	+0.057

4.6×10^6 (expt. 1) or 6×10^6 (expt. 2) "Daudi" [5] cells were incubated, in the "direct" fluorescence test (see *Methods*, (1)), with 0.05 ml of 1:15 diluted anti-IgM or anti-IgA fluorescent antisera. Control tubes ("blanks") contained two different "negative" cell lines: "Ester" [6] in expt. 1, "Silfere" [9] in expt. 2.

Each assay was run in duplicate, but the results were practically identical.

The "indirect" method has no such problem, since the fluorescent antiserum used is always the same, and only the "intermediate" serum varies. In Figures 1, 2, 3, and 4, titrations with scalar dilutions of nonfluorescent serum by the "indirect" method are shown. The same amount of fluorescent antisera was used in all assays of each group.

The reactivity of biopsy cells from a human reticulum cell sarcoma with isoantiserum is demonstrated in Figure 1. The procedure used in this test involved

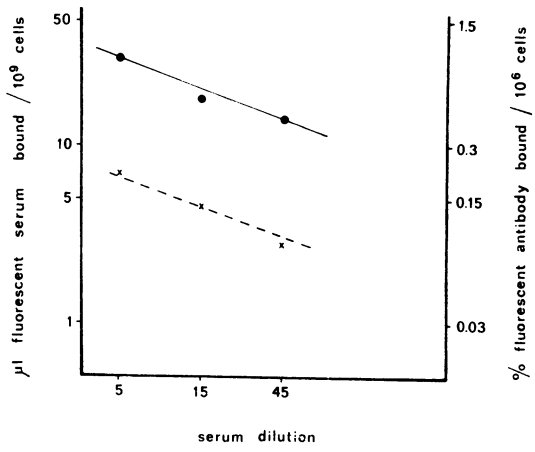

FIG. 1.—Binding of fluorescein-coupled anti-human IgG reagent by human reticulum cell sarcoma biopsy preincubated with different dilutions of isoantiserum. (O—O) Serum from a subject (Moulène) who received multiple transfusions; (X---X) serum from a control, nonisoimmunized subject (Ingegerd). In both cases, 1:30 diluted fluorescein-coupled goat anti-human IgG was used.

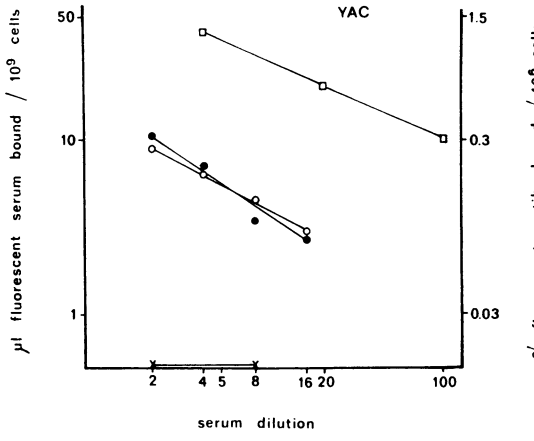

FIG. 2.—Binding of fluorescein-coupled anti-IgG reagent by YAC cells preincubated with mouse isoantiserum and rabbit antimouse serum. (X—X) Control (A/Sn mouse) serum, followed by 1:15 diluted goat anti-mouse fluorescein-coupled γ-globulin. The cells, after the reaction with the fluorescent antibodies, were lysed by freezing and thawing, and the precipitate at 0.4 saturation of ammonium sulfate was used for subsequent proteolysis and solubilization.

(O—O) ACA anti-A isoantiserum, followed by 1:15 diluted goat anti-mouse fluorescein-coupled γ-globulin. The precipitation with ammonium sulfate, as described above, was then carried out.

(□—□) Rabbit anti-mouse serum, followed by 1:15 diluted goat antirabbit fluorescein-coupled γ-globulin. The precipitation with ammonium sulfate, as described above, was then carried out.

(●—●) ACA anti-A isoantiserum, followed by 1:15 diluted goat antimouse fluorescein-coupled γ-globulin. Precipitation with ammonium sulfate was omitted, but step 2 of *Methods* was strictly followed.

ammonium sulfate precipitation for the elimination of heme. Suspensions of YAC cells—which do not contain erythrocytes—were processed with and without precipitation by ammonium sulfate and, as seen in Figure 2, in the parallel tests identical results were obtained, thus validating the procedure.

Figure 2 also shows the titration curves of isoantiserum and heterologous serum on YAC target cells. It can be seen that the binding of the fluorescent anti-immunoglobulin is strikingly higher when the cells are reacted with the rabbit antimouse serum than with the isoantiserum, but that the slope of the titration curve is approximately the same for the two sera.

Figure 3 shows instead that the slope is significantly lower if the serum used is directed against a smaller number of all isoantigen specificities. Table 2 lists the

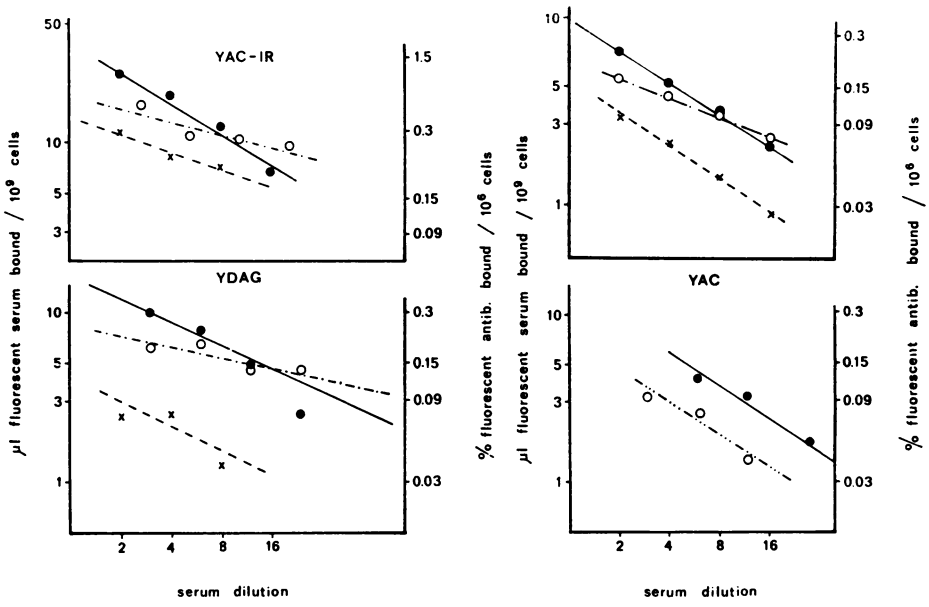

FIG. 3.—Titration curves of two isoantisera on various mouse cells. (×---×) Control (A/Sn mouse) serum; (O—O) ACA anti-A serum; (O-···-···-O) ACA anti-A serum, previously absorbed with membrane preparation of YAA cells; (O-·-·-O) (C57BL × DBA/2) F_1 anti-C3H serum. In all cases, 1:15 diluted fluorescein-coupled goat anti-mouse γ-globulin was used.

variations in the slope of the titration curves for different kinds of cells, tested with various sera. It can be seen that a maximal slope, generally around 0.6, is obtained both with ACA anti-A and with heterologous serum. With sera directed against a narrow range of H_2-antigens (anti-4, 10, 13, 31 and anti-1, 11, 25,32) significantly lower slopes were found. Moreover, when anti-1, 11, 25, 32 serum was tested against YDAG ascites cells, of A × DBA/2 F_1 genotype being thus heterozygous with respect to isoantigens 1, 11 and 25 and lacking isoantigen 32,

TABLE 2. *Variation in the slope of the titration curves.*

	Immune Sera			
Target cells	ACA anti-A	(C57BL × DBA/2)F_1 anti-C3H	(C57BL × C3H)F_1 anti-DBA/2	Rabbit anti-mouse
YAC A/Sn	0.66, 0.53 0.57, 0.60	0.44, 0.40	0.35	0.44, 0.54
YAC-IR A/Sn	0.54, 0.61	0.31, 0.39	0.40	0.60
A/Sn Lymph node	0.57			
YDAG (A/Sn × DBA/2)F_1	0.51	0.20		
YDYA (ABY × DBA/2)F_1			0.18	

For experimental details, see text. Each value indicated is the result of a single experiment and indicates the slope of the titration curve in the log vs. log scale.

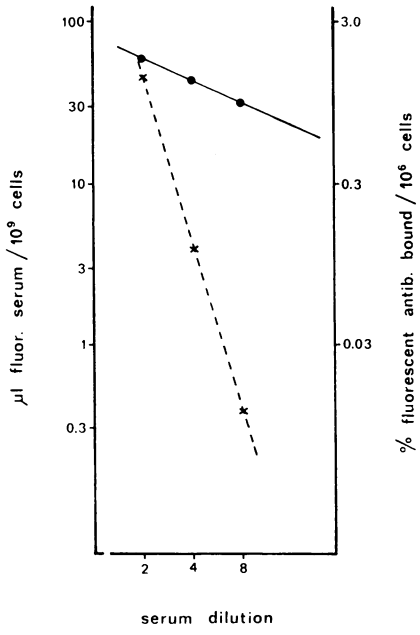

FIG. 4.—Binding of fluorescein coupled anti-mouse IgG reagent by Ha3 cells pretreated with different dilutions of anti-Moloney virus mouse serum. (O—O) Anti-Moloney virus-specific mouse (A/Sn) antiserum; (X---X) control (A/Sn mouse) serum. In both cases, 1:15 diluted fluorescein-coupled goat anti-mouse γ-globulin was used.

the slope was further decreased. The possible influence of the cell size on the slope was investigated by using cells with equal genotype but different size distribution (e.g., YAC, YAC-IR, and A/Sn lymph node cells; Ha2 and Ha3 cells). No clear-cut relationship could be obtained. Absorption of ACA anti-A serum with a membrane preparation from YAA ascites tumor cells of A/Sn origin, kindly given to us by Dr. Yata, resulted in a marked lowering of the amount of fluorescent antibody bound, but the slope of the titration curve remained the same (Fig. 3).

In most of the cell lines investigated, the fluorescent serum uptake in the presence of control serum (i.e., serum from syngeneic mice or, for other cells, serum which at the fluorescence microscope was scored as negative) was less than one fifth of that seen with the immune serum. With YAC tumor cells, however, the binding in the presence of control serum was often relatively high. This fact, which was found to be slightly decreased by using control sera from individual syngeneic mice instead of pooled sera, or even by previously absorbing the control sera on other cells, sometimes makes the results obtained with immune sera on YAC cells rather difficult to interpret properly, especially when slope values are examined. The nonspecific absorption of control serum is not, on the other hand, constant even with YAC cells, but seems to be higher when the cells are collected soon after the inoculation (i.e., within 7 to 8 days) and decreases afterwards.

Reactivity of the Ha3 tissue culture cells with Moloney specific mouse antiserum is given in Figure 4. In this case also, the amount of fluorescent anti-IgG antibodies which bind after treatment with high concentrations of control serum is relevant; upon dilution of the control serum, however, this unspecific binding

decreases rapidly, while the binding mediated by the specific anti-Moloney antiserum remains relatively high. The same phenomenon can be observed also by visual examination at the fluorescence microscope. It is therefore inherent in the biological system used and cannot be attributed to a defect of the method described in the present paper. This difficulty can probably be overcome by using, instead of whole serum, purified α-globulins.

Compared to the microscopic evaluation of the fluorescence, this method suffers the drawback of the need for high numbers of target cells per sample. It has, however, two advantages: (1) It gives quantitative results; (2) it offers the possibility of evaluating independently, and in a strictly quantitative way, the influence of antiserum dilution upon the binding, this parameter probably being correlated to the density of antigen receptors on the cell surface.

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[1] Möller, G., *J. Exptl. Med.*, **114**, 415 (1961).

[2] Klein, G., and E. Klein, *J. Natl. Cancer Inst.*, **32**, 547 (1964).

[3] Goldstein, G., G. Klein, G. Pearson, and P. Clifford, *Cancer Res.*, **29**, 749 (1969).

[4] Fenyö, E. M., E. Klein, G. Klein, and K. Swiech, *J. Natl. Cancer Inst.*, **40**, 69 (1968).

[5] Klein, E., G. Klein, J. S. Nadkarni, J. J. Nadkarni, H. Wigzell, and P. Clifford, *Cancer Res.*, **28**, 1300 (1968).

[6] Nadkarni, J. S., J. J. Nadkarni, P. Clifford, G. Manolov, E. M. Fenyö, and E. Klein, *Cancer*, **23**, 64 (1969).

[7] Chuat, J.-C., L. Berman, P. Gunvén, and E. Klein, *Intern. J. Cancer*, **4**, 465 (1969).

[8] Snell, G. D., G. Hoecker, D. B. Amos, and J. H. Stimpfling, *Transplantation*, **2**, 777 (1964).

[9] Klugerman, M. R., *J. Immunol.*, **95**, 1165 (1966).

[10] Neurath, A. R., *Z. Naturforsch.*, **20b**, 974 (1965).